Caspofungin versus amphotericin B treatment of *Aspergillus fumigatus* in kidneys of chronically immunosuppressed infected mice

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Background: Caspofungin acetate has been approved for use in empirical therapy of presumed fungal infections in febrile, neutropenic patients, candidemia, esophageal candidiasis and invasive aspergillosis in patients who cannot tolerate or respond to other therapies. While caspofungin has been studied extensively in mouse survival models of aspergillosis, no study to date has documented the time or dose required to eliminate the detectable *Aspergillus fumigatus* fungal burden in the kidney of an established mouse infection model. **Aim:** The goal of this study was to determine the duration of treatment and dose of caspofungin required to reduce the *A. fumigatus* burden to the limit of detection in kidneys of challenged, immunosuppressed mice. **Materials & methods:** Dosing was initiated 24 h after infection, and amphotericin B (AmB) was used as a comparator. Immunosuppressed mice were infected intravenously with *A. fumigatus* and treated intraperitoneally for 14 days with caspofungin (1 or 2 mg/kg), AmB (0.5 mg/kg), or vehicle. Both survival and fungal burden in kidneys were assessed in cohorts by histologic and quantitative polymerase chain reaction analysis. **Results:** There were no survivors among infected, vehicle-treated mice by day 10 after challenge. At day 42 after challenge in the survival arm of the study, 60% of the mice treated with 2 mg/kg caspofungin, 50% treated with 1 mg/kg caspofungin and 30% treated with 0.5 mg/kg AmB survived. The overall trends for reduction of the kidney burden were similar between quantitative polymerase chain reaction and histologic exam. A total of 92% of animals in the groups receiving caspofungin (1 or 2 mg/kg/day) had no detectable evidence of residual infection on day 42, the last day of evaluation. **Conclusion:** These results indicate that caspofungin dosed at 1 or 2 mg/kg reduced the histologic signs of *A. fumigatus* and the kidney burden to the limit of detection in chronically pancytopenic mice. Caspofungin was at least as effective as AmB in both the magnitude and the rate of decrease in *A. fumigatus* kidney burden in this model.

The incidence of reported invasive fungal infections has risen mainly due to an expanding number of immunocompromised patients, improved diagnostic capabilities and an increased use of chemotherapeutics that cause immunosuppression. Safe and effective antifungal therapy options are limited. Amphotericin B (AmB) is a broad spectrum antifungal agent that has often been documented to induce anemia and renal toxicity with repeated usage at clinically approved doses [1]. Caspofungin inhibits β-1,3-D-glucan synthesis in fungi. Cell walls containing glucans are unique to fungi – glucans are not present in human cells and therefore in humans, mechanism-based toxicity is not an issue. Caspofungin and AmB have both demonstrated efficacy in animal models of disseminated and pulmonary aspergillosis [2–6]. Aside from prolongation of survival, these compounds can reduce the fungal burden in organs of *Aspergillus fumigatus*-infected mice relative to vehicle-treated mice [3]. However,

the treatment duration necessary to eradicate fungal burden from organs of *Aspergillus*-infected mice has not been studied.

Quantitative polymerase chain reaction (qPCR) is an established, reliable method of identifying *Aspergillus* in infected tissues or monitoring the progression of fungal infection in mice [3,7]. This methodology better reflects the fungal mass as compared with tissue colony-forming unit (CFU) counts [3]. In a direct comparison of CFUs with qPCR quantitation in kidneys, peak burden values from qPCR analysis were over 1000-fold higher than CFU values, and there was a direct correlation between maximal tissue burden measured by qPCR and the onset of significant mortality [3].

In this study, we used a 24 h delayed therapy, immunosuppressed murine model of disseminated *A. fumigatus* to determine the time required for caspofungin or AmB to reduce the kidney-fungal burden as determined by survival, tissue histology and qPCR.

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Materials & methods

Organism

A. fumigatus, MF5668 (ATCC 13073), was grown on sabouraud dextrose agar (SDA) at 35°C for 7 days. The conidia from one SDA slant were washed from the surface of the agar with 5 ml of sterile physiologic saline plus 0.01% Tween 20. The conidia concentration (conidia/ml) was determined by counting with a hemacytometer and adjusted by dilution in sterile saline. The CFU count was determined by serial dilution of the spore suspension tenfold and plating on SDA plates.

Mice

Female CD-1 mice (Charles River Laboratories) weighing 23 to 27g (6–7 weeks) were used in these studies. All procedures were carried out with the highest standards for the humane handling, care and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee. Procedures for the care and use of research animals at Merck meet or exceed all applicable local, national and international laws and regulations.

Animal protocol

All time points are relative to day 0, the time of inoculation. On day -3, all mice (total n = 225) were administered 0.5 ml Cytoxan® at 12 mg/ml (6 mg/mouse) orally. Pancytopenia was then maintained by per oral administration of 0.5 ml cytoxan at 4 mg/ml (2 mg/mouse) on day 1 after challenge for all mice, and subsequently, every third day for the duration of the study. Immune suppression was monitored by differential counts in representative cytoxan-treated and normal CD-1 mice. On day 0, mice were infected intravenously in the lateral tail vein with 0.2 ml of spore suspension (3×10^4 conidia/mouse) and therapy was administered 24 h after challenge for 14 days. Mice were randomly allocated to one of four groups:

- Vehicle (sterile dH$_2$0)
- Caspofungin 1 mg/kg
- Caspofungin 2 mg/kg
- AmB 0.5 mg/kg

Caspofungin or AmB was solubilized and diluted serially in sterile dH$_2$0. The dosing regimen for all animals was intraperitoneally, once a day for 14 days.

To monitor survival, cytoxan-treated control mice that were infected and treated with vehicle, caspofungin at 1 or 2 mg/kg, or AmB at 0.5 mg/kg (n = 10/group) were included in a separate survival arm of the study. An additional five mice were cytoxan treated but sham infected to ensure there were no superinfections related to the immunosuppression, under the same conditions as the test mice.

To determine histologic scores and conidial equivalents (CE) at specified time points (days 1, 3, 5, 8, 15, 22, 29, 35 and 42) following challenge, five to ten mice from each group were euthanized by CO$_2$ inhalation and kidneys were removed with half of each kidney sectioned and placed in buffered formalin for histologic sectioning and half snap-frozen on dry ice for qPCR analysis.

Histology protocol

Kidney halves designated for histology were harvested and saved in formalin until they were embedded in paraffin and sectioned longitudinally at approximately 5 µ intervals. Once the kidney sections were placed on the slides, they were stained with hematoxylin and eosin (H&E) and gomori methenamine silver (GMS). One section/mouse was examined microscopically for the presence of hyphae typical of *Aspergillus*. The investigator was blinded as to the treatment groups . A subjective score from 0 to 4+ (**Figure 1**) was assigned to each sample as follows:
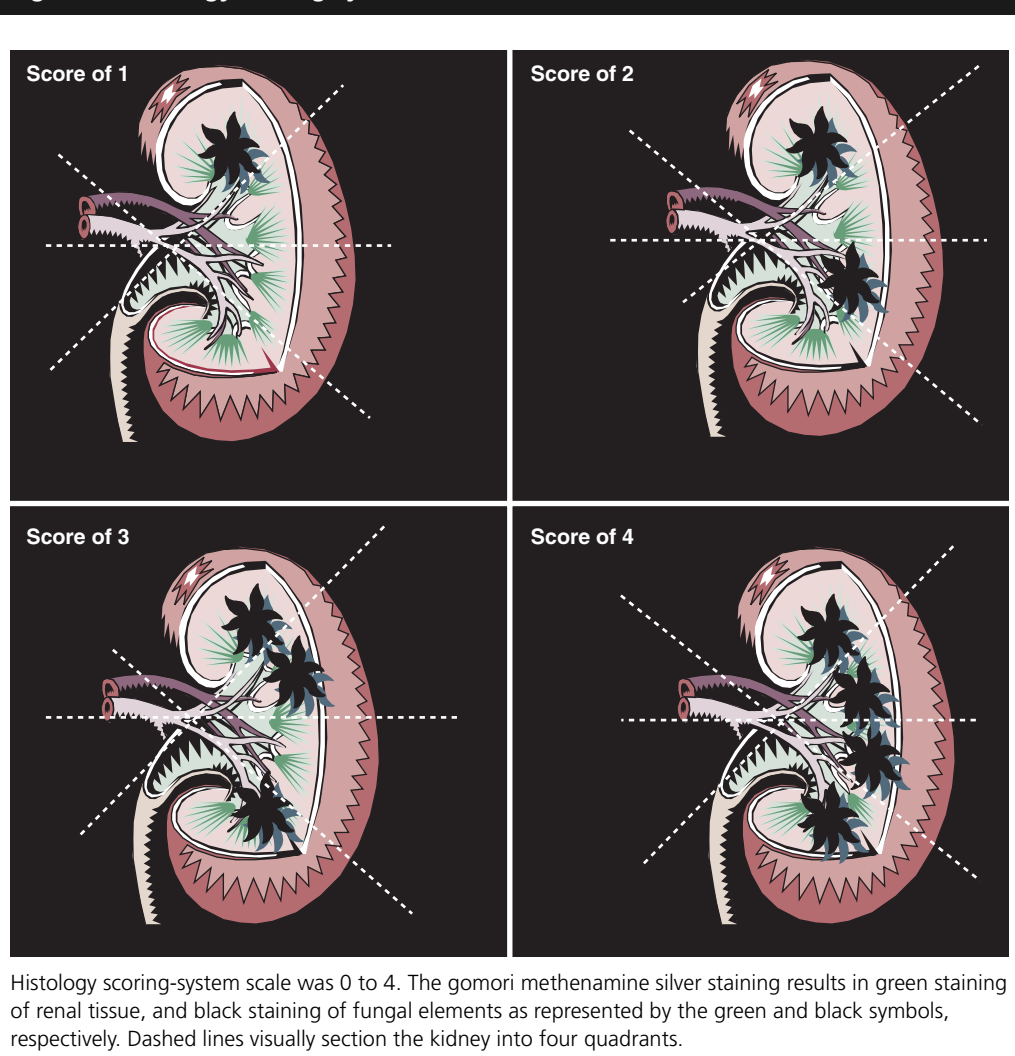
- 0 being negative – no hyphae
- 1 being < ¼ of organ infected
- 2 being ¼ to ½ infected
- 3 being ½ to ¾ infected
- 4 being > ¾ infected

Quantitative PCR protocol

Preparation of primary and secondary homogenates, isolation of DNA, sequence of primers and probes used to detect the *A. fumigatus* 18S rDNA target sequence, and conditions for the qPCR reaction have been described [3]. The method for generating standard curves based on *A. fumigatus* conidia spiked into naive, uninfected kidneys, and the conversion of qPCR tissue values into conidial equivalents/g of kidney tissue have also been reported [3].

Normalization for DNA recovery was performed by quantifying a nonmurine, nonfungal DNA sequence which was added to all samples prior to homogenization. A plasmid bearing a 3 kb fragment encompassing the *Eimeria tenella PKG* gene was spiked into the saline added to organs in

Figure 1. Histology scoring-system scale.

Histology scoring-system scale was 0 to 4. The gomori methenamine silver staining results in green staining of renal tissue, and black staining of fungal elements as represented by the green and black symbols, respectively. Dashed lines visually section the kidney into four quadrants.

the Whirl-Pak bags; after homogenization and DNA isolation, samples were analyzed by qPCR with the following primers and probes specific for the parasite gene sequence:

- Sense amplification primer: 5′-AGGGCTTTGCTGCACGAC-3′

- Antisense amplification primer: 5′-TCCAC-CTCGGGACTGTTTG-3′

- Hybridization probe: 5′-VIC-TGCTACT-GTTGCAGACCGCCGCT-TAMRA-3′

PCR reactions and data analysis were performed as for the *A. fumigatus* 18S rDNA target. A standard titration curve of signal as a function of plasmid copy number was constructed by serially diluting the plasmid in DNA from naive mouse organs and adding directly to reaction tubes. The percent recovery of *A. fumigatus* DNA in each organ sample was based on plasmid recovery [8,9].

Data analysis

The histologic scores were handled as categorical via multinomial model fitting. The statistical model fit provided a series of estimated probabilities for each possible score category. The products of the scores × their respective probabilities were summed to arrive at a weighted mean score. Inference for mean scores based on these models was obtained through case-based (nonparametric) bootstrapping [10] where the resampling scheme follows the group-by-time points structure of data.

The CE threshold values are handled as censored, so that methods could be applied in the form of a log normal accelerated failure time model. Based on the model fit to all of the CE data, each of the three therapy groups are compared with the sham control at time points at days 3, 5 and 8, as these are days when sufficient sham sample sizes existed. The log normal

accelerated-failure time-model approach is also taken for the survival data, since subjects alive at day 42 are considered censored.

The R environment was used for all calculations and the production of graphs. The built-in Trellis-style library (lattice/grid), the boot library, and the Modern Applied Statistics with S (MASS) library [11,12] provided function commands in addition to the standard R system calls [13,14].

Results
Survival
Data from the survival arm of the study shows 100% mortality in vehicle-treated mice by Day 10 (Figure 2). At the end of the 14 day treatment period, the group dosed with 0.5 mg/kg AmB showed 40% survival, the 1 mg/kg caspofungin group showed 50% survival and the 2 mg/kg caspofungin group had 60% survival. By day 42 (28 days after the end of therapy), the AmB group had dropped to 30% survival, but caspofungin therapy at 1 or 2 mg/kg/day maintained 50 and 60% survival, respectively.

Histologic assessment of fungal burden
The *A. fumigatus* kidney burden based on histology is shown in Figure 3. On day 3, the mean histology score from kidneys of vehicle-treated mice was 0.9 ± 0.2. Treatment with caspofungin at 2 mg/kg yielded a mean histology score of

0.2 ± 0.2. Kidneys from caspofungin 1 mg/kg and AmB 0.5 mg/kg yielded scores of 0. All three treatment groups demonstrated significantly lower scores than vehicle-treated mice ($p < 0.002$).

On day 5, the vehicle-treated histology mean score was 2 ± 0.4. The caspofungin 2 mg/kg score remained at 0.2 ± 0.2 while, caspofungin 1 mg/kg scores increased to 0.2 ± 0.2. These two group scores were significantly lower than vehicle-treated scores ($p < 0.001$). Scores from kidneys of mice treated with AmB increased to 1.8 ± 0.9, which was not different from vehicle-treated scores.

On day 8, 60% of the vehicle-treated mouse kidneys had histology scores of 0; therefore the mean score was low (0.2 ± 0.2). None of the treatment-group scores were significantly different from vehicle-treated scores due to this low mean-vehicle score.

After termination of treatment, the group of mice that had received 2 mg/kg caspofungin demonstrated complete reduction of *A. fumigatus* from kidney tissue by day 15, which was confirmed on day 22. After day 22, none of the animals receiving 1 mg/kg of caspofungin had histological evidence of residual *A. fumigatus* in kidney sections examined. Kidney sections from AmB-treated animals did not reach this limit of histological detection until day 35 postinfection.

Quantitative PCR fungal burden
On day 3, the mean kidney burden of vehicle-treated mice was 5.1 $\log_{10}$ CE/g kidney (Figure 4). Kidneys from mice treated with caspofungin at 2 or 1 mg/kg showed a mean burden of 2.9 and 3.4 $\log_{10}$ CE/g kidney, respectively. These values are significantly lower than the burden in kidneys of vehicle-treated mice ($p < 0.04$). On day 3, animals from the AmB treatment group had a mean value of 4.2 $\log_{10}$ CE/g kidney, which was not significantly different from vehicle controls.
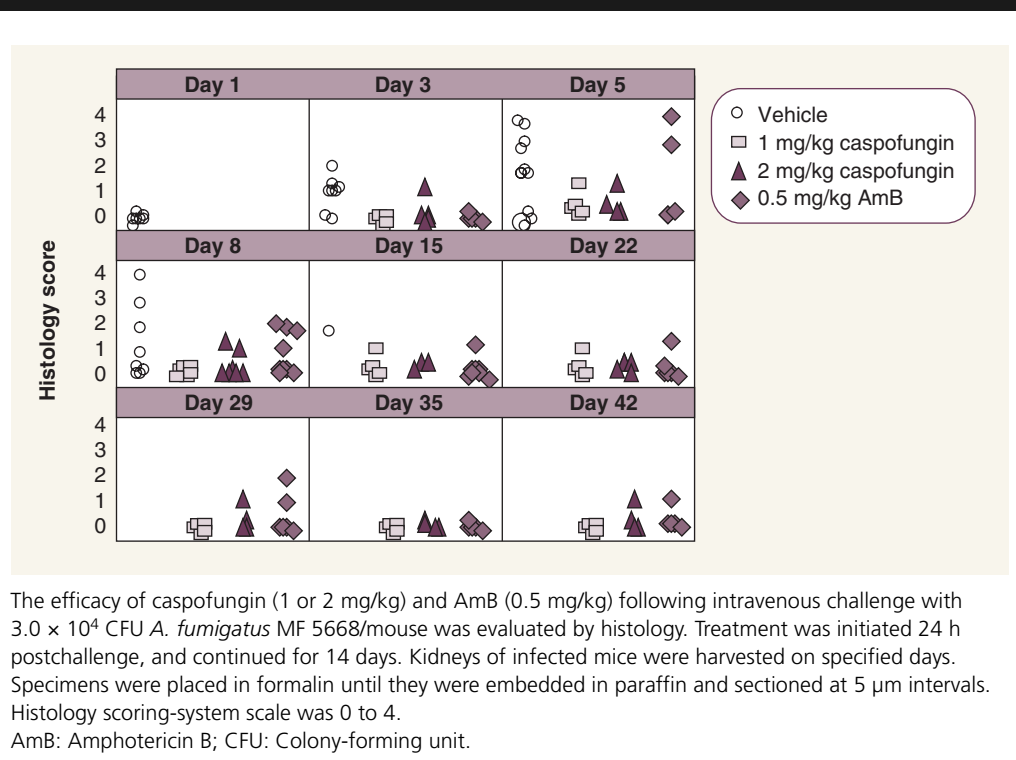
Kidneys from vehicle- and AmB-treated mice on day 5 showed a mean score approaching 6 $\log_{10}$ CE/g kidney. Caspofungin treatment of either 2 or 1 mg/kg yielded $\log_{10}$ CE/g kidney burdens of 2.8 or 2.3, respectively, both significantly lower than the value for vehicle-treated animals on day 5 (5.91 $\log_{10}$ CE/g kidney; $p < 0.001$). eight days postinfection, kidneys from all three treatment groups were significantly different from vehicle (5.51 $\log_{10}$ CE/g kidney; $p < 0.02$) with values of 2.9, 2.1 and 4 $\log_{10}$ CE/g kidney, respectively, for caspofungin 2 mg/kg, 1 mg/kg and AmB 0.5 mg/kg.

Figure 2. Survival over time following intravenous challenge with 3.0 x 10⁴ CFU of *A. fumigatus* MF5668.
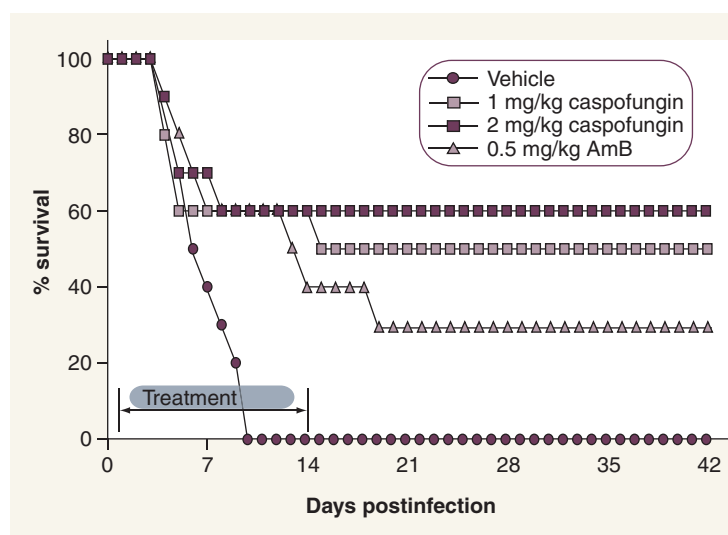
Mice were treated with vehicle, 1 or 2 mg/kg caspofungin, or 0.5 mg/kg AmB, beginning 24 h postchallenge, and continuing for 14 days. Survival was evaluated for 42 days. AmB: Amphotericin B; CFU: Colony-forming unit.

Figure 3. The efficacy of caspofungin (1 or 2 mg/kg) and AmB (0.5 mg/kg) as determined by histology.

The efficacy of caspofungin (1 or 2 mg/kg) and AmB (0.5 mg/kg) following intravenous challenge with 3.0 × 10^4 CFU *A. fumigatus* MF 5668/mouse was evaluated by histology. Treatment was initiated 24 h postchallenge, and continued for 14 days. Kidneys of infected mice were harvested on specified days. Specimens were placed in formalin until they were embedded in paraffin and sectioned at 5 µm intervals. Histology scoring-system scale was 0 to 4.
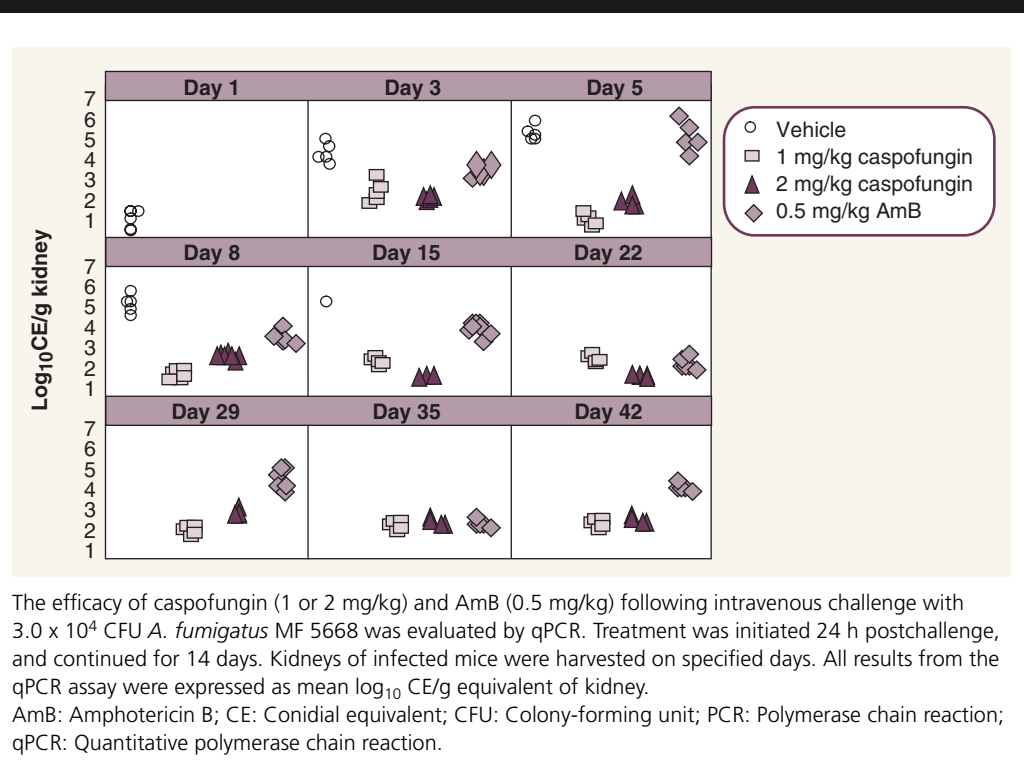AmB: Amphotericin B; CFU: Colony-forming unit.

Caspofungin administered at 2 mg/kg reduced the kidney burden of *A. fumigatus* to the limit of qPCR detection, which was 8 days after termination of treatment; caspofungin at 1 mg/kg required 28 days to reach the same end point (**Figure** 4). The qPCR data at day 35 showed reduction of fungal burden to the limit of detection in 60% of the mice treated with AmB.

Expert commentary & conclusion

Evaluation of the *in vitro* activity of caspofungin against filamentous fungi, including *A. fumigatus*, can be challenging. Interestingly, caspofungin does not give 100% inhibition of growth in traditional minimum inhibitory concentration (MIC) assays and there is no reduction in CFU in time–kill kinetic studies. However, caspofungin is highly effective clinically and in animal models of aspergillosis. This efficacy is attributed to the altered morphology of treated hyphae that are distorted, with stubby, thickened, highly branched germ tubes. Exposure of growing germlings to caspofungin leads to lysing of cells at tips and branch points, but minimal activity against subapical cells. There are many unanswered questions about the effect of caspofungin on *A. fumigatus*. However, altered methodology such as minimum effective concentrations

(MECs) and qPCR have been established to provide a more sensitive measure of echinocandin activity. Thus, this study was designed to evaluate the ability of qPCR to measure the time necessary to reduce the fungal burden to the limit of detection from a target organ in an invasive model of aspergillosis in the mouse. Results from this model helped provide some of the preclinical basis for evaluation of caspofungin in human invasive aspergillosis [5]. The 24 h delayed therapy model was used to simulate treatment of an existing infection and caspofungin AmB had shown efficacy in prior studies with this model [5]. The dosage of 1 mg/kg/day was used as it approximates the human dosage (70 mg/day) and drug plasma exposure (mouse area under the curve $[AUC]_{0-24}$ 83 µg*hr/ml, versus human AUC_{0-24} 118 µg*hr/ml). The MEC for caspofungin against *A. fumigatus* was 0.2 to 0.4 µg/ml; therefore the above exposures provide adequate coverage over this MEC.

Caspofungin (1 or 2 mg/kg) effectively prolonged survival in mice witgh induced, disseminated infection of *A. fumigatus*. This improved survival was maintained for 28 days beyond the end of therapy. Kidney burden was evaluated by both histology scores and qPCR. During the 14 days of therapy, qPCR appeared to detect burden that was

Figure 4. The efficacy of caspofungin (1 or 2 mg/kg) and AmB (0.5 mg/kg).

The efficacy of caspofungin (1 or 2 mg/kg) and AmB (0.5 mg/kg) following intravenous challenge with 3.0 x 10⁴ CFU *A. fumigatus* MF 5668 was evaluated by qPCR. Treatment was initiated 24 h postchallenge, and continued for 14 days. Kidneys of infected mice were harvested on specified days. All results from the qPCR assay were expressed as mean $\log_{10}$ CE/g equivalent of kidney.
AmB: Amphotericin B; CE: Conidial equivalent; CFU: Colony-forming unit; PCR: Polymerase chain reaction; qPCR: Quantitative polymerase chain reaction.

not observed in histologic sections. This was particularly evident in the day 8 samples for both caspofungin-treated groups as compared with vehicle-treated groups. On day 8 the mean histology scores of the caspofungin groups were not different from vehicle due to the low vehicle scores. The day 8 vehicle-treatment scores were low due to a score of 0 for 60% of kidney sections. However, by qPCR, the fungal burden was significantly higher (5.6 $\log_{10}$CE/g kidney) than caspofungin-treated groups. The kidneys used to determine the histology scores and qPCR burdens were bisected longitudinally, with half of each submitted for histology and half for qPCR. The histology half was embedded in paraffin and sliced in multiple 5 μ-thick sections, with only one section per kidney read by the pathologist. In contrast, the entire half submitted for qPCR was homogenized and samples were taken at random from the homogenate. The discrepancy is likely related to the difference in sensitivity of evaluating one thin portion of the organ as compared with samples from a homogenate.

Following termination of therapy, kidneys from mice that had received 2 mg/kg caspofungin were free of fungal burden by histological exam by day 15; this group remained free except for one animal on day 29 and one on day 42, each with a score of 1. This was essentially the same profile seen with qPCR. In each instance, the individual mouse

kidney half with a histology score of 1 had a qPCR score above the limit of detection, suggesting that these animals did not respond as well to therapy as the other treated mice in the same group. This further validates qPCR as a useful tool for measuring kidney burden of *A. fumigatus*. In kidneys where there was detectable burden by qPCR but not by histology, it was felt that the use of a sample from a whole-organ homogenate, combined with the inherent sensitivity of qPCR (detection of 0.1 conidial equivalents/mg equivalent of kidney), provided higher sensitivity compared with discrete histologic sections. This comparison of methods was similar for the caspofungin 1 mg/kg treatment groups, which showed all mice free of *A. fumigatus* by histology on day 28, but a low rebound on days 35 and 42 by qPCR. Kidneys from AmB-treated mice continued to show histologic evidence of fungal burden until day 35, with a slight rebound on day 42. By qPCR, fungal burden in kidneys of mice treated with AmB did not reach the limit of detection over the time period tested in this study.

In summary, kidney fungal burden appeared to be reduced to the limit of histologic detection by day 15 with caspofungin 2 mg/kg, day 28 with caspofungin at 1 mg/kg and day 35 with AmB. The overall trends of kidney fungal load reduction were similar between qPCR and tissue histology. A total of 92% of animals in the caspofungin

Highlights

• Caspofungin dosed intravenously at 1 or 2 mg/kg significantly decreased both histologic and quantitative polymerase chain reaction evidence of Aspergillus fumigatus infection.
• A total of 92% of animals receiving 1 or 2 mg/kg of caspofungin showed no evidence of residual infection on day 42.

1 mg/kg and 2 mg/kg group had no evidence of residual infection, based on histology and qPCR analysis, on day 42, the last day of evaluation. These results indicate that caspofungin dosed at 1 mg/kg or 2 mg/kg reduced histologic signs of *A. fumigatus* and significantly reduced the kidney fungal burden in chronically pancytopenic mice, respectively.

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